3736

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Leif ANDERSSON, et al.

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Filed: November 30, 1999

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For: METHODS FOR ANALYZING ANIMAL PRODUCTS

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Assistant Commissioner of Patents
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Sir:

Applicants are enclosing a certified copy of **Great Britain Application No. 9711214.8**, filed May 30, 1997 and **Great Britain Application No. 9801990.4,** filed January 31, 1998. These documents provide a basis for Applicants' claim for priority, which was made upon the filing of the above-identified patent application in the U.S. Patent and Trademark Office on November 30, 1999.

No fee is believed necessary with this submission. However, should the U.S. Patent and Trademark Office determine that additional fees are due upon the filing of this priority document, please charge any such fees to the undersigned's Deposit Account No. 02-0375.

Respectfully submitted,

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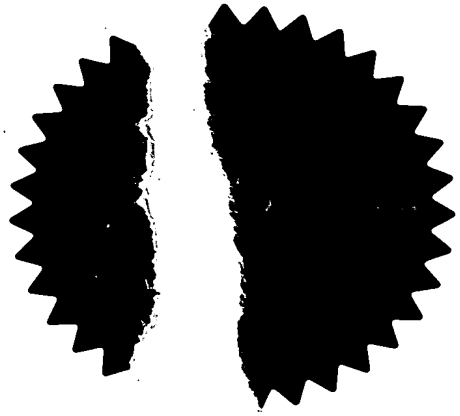

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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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Patents ADP number *(if you know it)* 07221575001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — SCREENING METHOD

5. Name of your agent *(if you have one)*

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SCREENING METHOD

The present invention relates to methods of screening pig nucleic acid to determine pig genotype with respect to coat colour, and to kits for use in carrying out such methods.

Pig breeds show a variety of coat colours and these are often associated with particular production characteristics. For example, white is the predominant coat colour among European commercial breeds e.g. Large White and Landrace, and these breeds are associated with larger litters and good mothering ability. However, there are a number of commercially important coloured breeds, demonstrating a number of colours and combinations. The Duroc, associated with meat tenderness, is red, the Pietrain, a heavily muscled animal which produces a very lean carcass, is spotted, and the Hampshire, also heavily muscled, is black with a white saddle over its shoulders. In addition, there may be other useful local breeds which have traits of potential commercial interest, and which are coloured. For example, the Chinese Meishan breed has been imported into Europe and the US because of its very large litter size. The European Wild Boar is brown when adult and striped when juvenile, and this breed is utilised to satisfy consumer demand for traditional meat products. It is also claimed that other local breeds or landraces are important because of their adaptation to local environments, e.g. temperature, endemic diseases and local feedstuffs.

Coat colour is important to the pig industry for a number of reasons. Firstly, gross variation in appearance (i.e. a range of coat colours) of pigs claimed to be genetically consistent for traits other than coat colour can lead to questions about the consistency and quality of the animals in the mind of pig-producers. Thus, the

coat colour of the pig is often used as a trademark of the breed and the breeders want to ensure that their animals breed true for colour. For example, in several markets, local, traditional, coloured breeds are marketed for their meat quality or in terms of the production system used to rear them. However, this is not a trivial task since the coat colour is controlled by a number of genes. The inheritance is also complicated by the presence of dominance and interaction between genes. There is also an application in the assessment of the purity of the genetics of traditional breeds used as the basis for modern synthetic lines and the confirmation of the derivation of the latter.

Secondly, in a number of markets there is a preference for white skinned meat. This is due to the fact that pork is often marketed with the skin still attached, and skin from coloured pigs, even if dehaired, can still exhibit colour, which can lead to negative perception by the consumer partly, since the surface of the meat may appear to be spotted by mould. It is therefore necessary in these markets to remove the skin from such carcasses, entailing additional cost. For example, in the US, coloured carcasses are associated with approximately 1% skin defects requiring dehairing and skinning to remove pigment. As a result of this, coloured pig carcasses are generally discounted.

One example of the problem concerns the presence of black pigmented spots occurring in production animals that are crossbreds between a white and a pigmented line. This may occur because the dominant white gene inherited from the white breed is not always fully dominant in the heterozygous condition which occurs in this cross. A possible solution to this problem would be to ensure that the production animals are homozygous for the recessive red allele present in breeds such as the Duroc. In this case the pigmented spots would be red instead of black

and much less conspicuous. To achieve this one needs to breed the recessive red allele to homozygosity in both the white and pigmented line used for cross-breeding. However, this would be very difficult using phenotypic selection as selection for a red background colour in a white line could only be accomplished with very expensive progeny testing schemes.

In addition, pig breeders would like to be able to be in a position to ensure consistency in breeding populations. Breeders may wish to ensure that progeny produced by breeding crosses were always white. Alternatively, a breeder of Duroc or Hampshire pigs may wish to ensure that breeding crosses always produced the characteristic Duroc or Hampshire colouring. Traditional animal breeding practices have in the past, been used to attempt to eliminate untypical colour from pig lines. For example purebred breeders must submit potential boars for progeny testing in order to demonstrate that they are suitable for inclusion in the breeding herd. This procedure incurs significant cost, including the substantial delay to confirm sufficient matings and progeny have been produced before the animal can be used commercially.

Therefore, selection based on a diagnostic DNA test for mutations in coat colour genes would be a major advance compared with phenotypic selection.

Coat colour is determined by the action of a number of different genetic loci. For example, the gene determining whether a pig is white or coloured is designated *I* (for inhibition of coat colour). The version of the gene preventing the expression of any colour (*I*) is dominant to that which allows colour to develop (*i*). Traditional selection for white animals has reduced the frequency of *i*, but it still remains in the population in white heterozygous carrier animals.

Recently, a genetic difference in the *KIT* gene in pigs was found to be involved with this aspect of coat colour determination which allowed the development of methods of distinguishing between alleles at this locus. However, animals which carry two copies of the recessive allele, *i*, at this locus have non-white coat colours (Johansson-Moller *et al*, *Mamm. Genome*, 7:822-830 (1996), PCT patent publication no. WO-A-97/05278). Pigs of this type can be all one colour, such as the Duroc which is red, or have combinations of colours, particularly spotted or banded patterns, such as the Pietrain and Hampshire respectively. Many other combinations are possible and are observed.

The non-white colour in such animals may be varying shades of red or black. The type of colour expressed is determined by the action of a second gene which is designated *E* (for extension of coat colour). Based on the literature, animals which contain the *E* version of the gene are completely black, and this version of the gene is dominant to that which results in red coat colour (*e*). Patched or spotted animals, such as the Pietrain breed, contain a third version of the gene designated E^{p}. This version of the gene is dominant to *e* but not to *E*. For example, black animals may have the genotype, *iiEe*, *iiE*E^{p} or *iiEE*. A black sow and a black boar which were both heterozygous at the *E* locus and which were of the genotype *iiEe* would produce both black and red piglets in the ratio 3:1. The black piglets would be *iiEE* or *iiEe* and the red piglets would be *iiee*.

The density and coverage of coat colour and the position of bands of white are determined by additional loci, which are not considered here as we are concerned with the type of non-white coat colour. For example, the Hampshire breed is background black with a white band across its shoulders, the width of this band may vary, however, the colour should be black. There is evidence

that some Hampshire animals are derived from herds that have been crossed previously with red breeds, such as the Duroc. In this situation, the red version of the gene can be maintained silently in the heterozygous state. When two heterozygotes are crossed 25% of the offspring will contain red. In some cases such pigs will have the appearance of the Duroc breed, being solid red, however, in other cases, the animals will have the white band inherited from the Hampshire and have the appearance of red Hampshires. It is the presence of the atypical coat colour rather than the pattern that is important in this situation.

The extension locus is known in other species of domestic animals, such as the horse, where *e* is associated with chestnut colour (Adalsteinsson, *J. Hered.* **65**: 15-20 (1974)), cattle, where *e* is associated with recessive red coat colour (Klungland *et al*, *Mammalian Genome* **6**: 636-639 (1995)), the fox, where *e* interacts with a second locus termed agouti to overide non-red colours (Vage *et al*, *Nature Genetics*, **15**:311-315 (1997)) and the mouse, where *e* is associated with recessive yellow (Robbins *et al*, *Cell*, **72**: 827-834 (1993)). The extension locus encodes the alpha melanocyte-stimulating hormone receptor (αMSHR). It has been shown that recessive alleles at this locus do not express a functional αMSH receptor (Robbins *et al*, *Cell*, **72**: 827-834, Klungland *et al*, *Mammalian Genome* **6**: 636-639 (1995)) and these workers have identified mutations in the sequence of the αMSHR gene in these species associated with different coat colours.

Classical segregation analyses have identified a minimum of three alleles at the pig extension locus: *E* for uniform black, E^p for black spotting and *e* for uniform red (Ollivier and Sellier, *Ann. Génét. Sél. Anim.*, **14**:481-544, (1982)). The dominance relationship among the three

alleles is as follows $E>E^{p}>e$. We have now found that these coat colour variations are associated with sequence polymorphism in the αMSHR gene in the pig. We have analysed the DNA sequence of this gene using samples from the following breeds with different coat colour: Wild Boar which is wild type coloured, Meishan and Hampshire which carry alleles for uniform black (E), Pietrain and Large White which carry alleles for black spotting (E^{p}) and Duroc which is uniform red (e). In Large White the patches or spots of colour that might be expected due to the presence of the E^{p} allele are hidden as this breed also carries the dominant white gene which prevents any expression of colour. Four different αMSHR sequences were obtained one from the Wild Boar, one from Meishan, one from Duroc and one found in Hampshire, Pietrain and Large White. We have designated the allele found in the Wild Boar as E^{+} and assume that the presence of this allele is necessary for the expression of the wild type colour. The E alleles for uniform black carried by Meishan and Hampshire pigs were associated with different αMSHR sequences. We have denoted these two alleles E^{m} and E^{h}, respectively. The DNA sequence associated with the allele for black spotting (E^{p}) was unexpectedly indistinguishable from that of uniform black in Hampshire (E^{h}). The similarity of the E^{p} and E^{h} alleles suggests that they are derived from a common origin. The absence of a unique differentiating modification in the sequenced part of the gene implies that the difference is likely to be in the upstream or downstream part of the gene, possibly in a regulatory element of the genes. Sequence comparisons of these regions from Pietrain and Hampshire or other breeds will provide information to develop kits specific for E^{p} and E^{h}. Alternatively, alleles of linked markers, such as microsatellite or AFLP markers, found to be in linkage disequilibrium with these alleles could be used to predict colour genotype. In conclusion we have found four different αMSHR sequences associated with five

different extension alleles i.e. E^+, E^m, E^b/E^p and *e*.

All of the DNA sequence differences identified in the αMSHR gene are single base pair changes. Some of these are silent, however, a number lead to changes in the amino acid sequence of the αMSHR protein. For example, the differences between *e* and the other alleles are two missense mutations in the coding sequence of the αMSHR gene. Importantly, the differences in the pig gene is different from that found in other species. The cattle and mouse *e* mutations are one base pair deletions (Robbins *et al, Cell*, **72**: 827-834 (1993); Klungland *et al, Mammalian Genome* **6**: 636-639 (1995), Joerg *et al, Genome* **7**: 317-318 (1996)), whilst the mutations identified here include a missense mutation (G844 changed to A) in a region which is conserved among human, mouse, cattle and horse gene sequences (Wikberg *et al*, WO 94/04674 (1994), Valverde *et al, Nature Genet.* **11**: 328-330 (1995), Robbins *et al, Cell*, **72**: 827-834 (1993), , Klungland *et al, Mammalian Genome* **6**: 636-639 (1995), Joerg *et al, Genome* **7**: 317-318 (1996), Marklund *et al, Mamm. Genome*, **7**:895-899 (1996)). The E^p group has a single missense mutation which leads to a change in another conserved region, which is only four amino acid residues away from a modification observed in the dominant allele found in the fox (Vage *et al*, (1997), *supra*). Finally, the Meishan allele (E^m) shows four amino acid changes in the protein. Two of these differences are in the same region of the gene which is altered in cattle.

Thus this finding has allowed us to develop methods of distinguishing between the alleles *e*, E^+, E^m and E^p/E^b, and thus for determining the genotype of individual pigs with respect to non-white coat colour.

Associations can be determined between extension locus genotype and microsatellite sequences which are linked to

the gene. A number of microsatellite markers have been located to the region of porcine chromosome 6 to which the extension locus has been mapped (Mariani *et al, J. Hered.*, **87**:272-276 (1996)) including S0035 (Archibald *et al, Mammalian Genome*, **6**:157-175 (1995)), S0016 (Coppetiers *et al, Animal Genetics*, **26**:327-330 (1995)), S0099 (Johansson *et al, J.Hered.*, **83**:196-198 (1992)) and SW2535 (Alexander *et al, Mammalian Genome*, **7**:368-372 (1996)).

Therefore, in a first aspect, the present invention provides a method of determining the coat colour genotype of a pig which comprises:

(i) obtaining a sample of pig nucleic acid; and

(ii) analysing the nucleic acid obtained in (i) to determine which allele or alleles of the αMSHR gene are present.

In one embodiment of this aspect of the invention the determination in step (ii) is carried out by determining the nucleotide sequence of the αMSHR gene and, in particular, is based on determining which missense mutation is present in the coding region of the gene.

In another embodiment one could first determine the association between microsatellite marker alleles linked to the αMSHR gene and particular alleles of the αMSHR gene. Thus, the determination in step (ii) would be based on identification of microsatellite marker alleles present in the nucleic acid sample.

In a second aspect, therefore, the present invention provides a method of determining the coat colour genotype of a pig which comprises:

(i) determining the association between one or more microsatellite marker alleles linked to the αMSHR gene and particular alleles of the αMSHR gene;

(ii) obtaining a sample of pig nucleic acid; and

(iii) analysing the nucleic acid obtained in (ii) to determine which microsatellite marker allele or alleles are present.

The determination of the alleles at the extension locus will indicate the background colour of the animal and in some cases the pattern of mixed colouration, i.e. spotting, but will not necessarily determine the coat colour of resulting progeny. This will be dependent on the genotype at other loci such as the dominant white locus, *I*. The genotype at the *I* locus can be determined separately as described in WO-A-97/05278.

Thus, suitably, the methods as described above may further comprise the step:

(iii)/(iv) determining the genotype of additional coat colour loci.

Suitably, the methods of the present invention will be carried out on pig genomic DNA, although pig RNA may also be analysed to determine the presence of absence of the *e* mutation. Any method identifying the presence of the specific sequence change may be used, including for example single-strand conformation polymorphism (SSCP) analysis, ligase chain reaction, mutagenically separated PCR, RFLP analysis, heteroduplex analysis, denaturing gradient gel electrophoresis, temperature gradient

electrophoresis, DNA sequence analysis and non-gel based systems such as Taqman™ (Perkin-Elmer).

Preferably, the determination carried out in step (ii) involves the use of PCR techniques, using an appropriate pair of primers. PCR, or polymerase chain reaction, is a widely used procedure in which a defined region of a DNA molecule can be amplified *in vitro* using a thermostable version of the enzyme DNA polymerase. Two known sequences that flank the region to be amplified are selected and priming oligonucleotides synthesised to correspond to these regions. If the primers are located sufficiently close together on the same piece of DNA, the region between them will be amplified. A polymerase chain reaction consists of a number of cycles of amplification. Each cycle begins with a denaturation step, typically at 94°C, in which two strands of the template DNA molecule are separated. The temperature is then dropped to a temperature at which the synthetic oligonucleotide primers can anneal to the template (typically 50-60°C). Through the high concentration of primers relative to template, the primers anneal to the template before template-template hybrids form. The annealing temperature is chosen such that annealing only occurs to the complimentary regions of DNA within the template, and not to other regions of imperfect complementarity. The temperature is then raised to 72°C, at which the thermostable DNA polymerase can extend the bound primer, thus producing the strand of DNA complementary to the template. Suitable pairs of primers which can be used in such PCR methods include:

MSHR Forward primer 1 sequence: 5′ CGG CCA TCT GGG CGG GCA GCG TGC- 3′

and

MSHR Reverse primer 1 sequence: 5′ CAG GAA ACA GCT ATG ACC GTC MTG CGG AGC TCC TGG CT- 3′

or

MSHR Forward primer 2 sequence: 5′ CGG CCA TCT GGG CGG GCA GCG TGC- 3′

and

MSHR Reverse primer 2 sequence: 5′ GGA AGG CGT AGA TGA GGG GGT CCA- 3′

or

MSHR Forward primer 3 sequence: 5′ GCA CAT CGC CCG GCT CCA CAA GAC- 3′

and

MSHR Reverse primer 3 sequence: 5′ GGG GCA GAG GAC GAC GAG GGA GAG- 3′

Preferably, the analysis of the amplified segment to determine the presence or absence of mutations that form the basis of the difference between alleles at the *E* locus will use restriction endonucleases. Restriction endonucleases are enzymes binding DNA at specific sequence motifs which then cleave at the same or a different sequence motif. Mutations that alter either the binding or cleavage motif can therefore be detected using a specific restriction endonuclease. Cleavage or non cleavage of amplified fragments can then be analysed through the use of size fractionation through electrophoresis on agarose or polyacrylamide gels or in capillaries.

The alleles carried by an animal can be designated as

either *e*, E^m or a member of the group E^+, E^p or E^h through the digestion of specific pig αMSHR amplification products with the restriction enzymes *BstUI* and *HhaI* (in separate reactions). Similarly digestion or non digestion upon treatment with restriction enzyme *Bsp*HI can be used to distinguish between the allele groups containing E^p and E^h or that containing E^m, E^+ and *e* respectively. Analysis of the fragments obtained from the digests on the basis of size allows the determination of the genotype. The preferred technique is gel electrophoresis.

In this technique, the digested fragments are separated in a supporting medium by size under the influence of an applied electric field. Gel sheets or slabs, such as agarose or agarose-acrylamide, are typically used as the supporting medium. The sample, which contains the restriction fragments, is added to one end of the gel. One or more size markers are run on the same gel as controls to permit an estimation of the size of the restriction fragments. The fragments are visualised by a DNA staining protocol, for example using the dye Ethidium Bromide.

Other possible techniques include non-gel systems such as TaqMan™ (Perkin Elmer). In this system oligonucleotide PCR primers are designed that flank the mutation in question and allow PCR amplification of the region. A third oligonucleotide probe is then designed to hybridize to the region containing the base subject to change between different alleles of the gene. This probe is labelled with fluorescent dyes at both the 5′ and 3′ ends. These dyes are chosen such that while in this proximity to each other the fluoresence of one of them is quenched by the other and cannot be detected. Extension by Taq DNA polymerase from the PCR primer positioned 5′ on the template relative to the probe leads to the cleavage of the dye attached to the 5′ end of the

annealed probe through the 5'nuclease activity of the Taq DNA polymerase. This removes the quenching effect allowing detection of the fluoresence from the dye at the 3'end of the probe. The discrimination between different DNA sequences arises through the fact that if the hybridization of the probe to the template molecule is not complete. i.e. there is a mismatch of some form, the cleavage of the dye does not take place. Thus only if the nucleotide sequence of the oligonucleotide probe is completely complimentary to the template molecule to which it is bound will quenching be removed. A reaction mix can contain two different probe sequences each designed against different alleles that might be present thus allowing the detection of both alleles in one reaction.

Although the TaqMan™ system is currently capable of distinguishing only two alleles, labelled probe primer sets could be developed in which the probes for certain target allele or alleles are labelled with a different fluorescent dye from non target alleles. For example, if one wished to confirm that a group of Duroc breed pigs carried only allele *e* one could have a probe present capable of detecting this allele labelled with one fluorescent dye and probes capable of detecting all the other alleles, E^+, E^p or E^m or E^h labelled with a second dye. Thus one would detect the presence of any non Duroc type alleles at this locus. Such probe sets could be designed and labelled according to the needs of the experiment.

Comparison of the αMSHR coding sequence from five porcine breeds with that of the Wild Boar revealed seven differences. One of these is present in the group comprising Hampshire, Large White and Pietrain. Two are present in the Duroc and four are present in the Meishan breed. Figure 1 shows a portion of the aligned sequence

containing the polymorphism detected in the four groups. For example, the two polymorphisms detected in red animals, are at nucleotide positions 608 and 844. The first of these shows a change from a C to a T. This results in an amino acid change of an alanine for a valine. However, this region of the protein is not particularly conserved between species and the amino acid is valine in the wild type allele of horse αMSHR. However, the Duroc sequence contains an A at position 844 while the other breeds all contain a G. This results in an amino acid change of an alanine for a threonine at the first position in the seventh transmembrane domain of the receptor protein, a region highly conserved in all species sequenced to date. This nucleotide change results in a restriction site polymorphism, as the replacement of G by A in position 844 removes the recognition site for the restriction enzyme *BstUI* (CGCG).

The codon for amino acid Ala 240 in alleles E^+ and E^D is altered in both alleles *e* and E^m but in different ways. In alleles E^+, E^n and E^D the sequence present is GGCGCGGCC at positions 841 to 849 which contains both a *Bst*UI site (CGCG) and a *Hha*I site (GCGC) and thus a fragment amplified from these alleles is susceptible to digestion at this position by both of these restriction enzymes. In allele E^m the DNA sequence is GGCGCAGCC thus a fragment amplified from this allele does not contain a site susceptible to digestion by *Bst*UI at this position. The susceptibility to digestion with *Hha*I remains. In allele *e* the DNA sequence of this region is GGCACGGCC thus there is no susceptibility to digestion by *Bst*UI or *Hha*I at this site.

Allele E^D as carried by the large white breed can be distinguished from E^+ through the amplification of a region of the porcine αMSHR gene containing nucleotide position 487 which differs between the two alleles. In alleles E^D and E^n nucleotide sequence between positions

482 and 487 is TCATGA which is susceptible to digestion by the restriction enzyme *Bsp*HI. However in the alleles E^{m}, E^{+} and *e* the nucleotide sequence is TCATGG and thus products amplified from these latter alleles are resistant to digestion by this enzyme

In a third aspect the present invention provides a method of determining the coat colour genotype of a pig which comprises:

(i) obtaining a sample of pig nucleic acid;

(ii) carrying out one or more PCR amplification cycles of the nucleic acid obtained in step (i) using at least one pair of suitable primers; and

(iii) carrying out RFLP analysis on the amplified nucleic acid obtained in step (ii) to determine the αMSHR genotype of the pig.

Where appropriate, preferred features of the third aspect of the invention are as for the first or second aspect, *mutatis mutandis*.

The skilled person will appreciate that since the polymorphisms discussed herein lead to a modified αMSH receptor protein, that this in itself can be used to determine a pig's genotype. Therefore, in a fourth aspect the present invention provides a method of determining the coat colour genotype of a pig which comprises:

(i) obtaining a sample of pig αMSHR protein; and

(ii) analysing the protein obtained in step (i) to determine the amino acid sequence at the variant positions associated with coat colour genotype.

The methods of the present invention can conveniently be carried out using kits, which comprise those reagents etc required to perform the tests. Thus, in a further aspect the present invention provides a kit for determining the coat colour genotype of a pig, comprising one or more reagents for analysing the αMSHR genotype of the pig. Preferably, the kit is adapted to be used with a sample of pig genomic DNA and the reagents are for use in one or more cycles of PCR using a pair of suitable primers, such as those described herein. In addition, the kit may comprise one or more reagents for use in carrying out RFLP analysis on an amplified sample of pig nucleic acid.

The Duroc breed is associated with improved eating quality of fresh pork (greater tenderness). In some situations retailers demand that the pig used for their supply contain at least a proportion of Duroc genetics. It is not possible to determine whether a white pig contains 50% Duroc genes as it enters the food chain. Breed specific DNA markers will provide a method of determining that such pigs contain the required level of Duroc genetics. Microsatellite markers may be suitable for this purpose. However, such markers are very variable and it may not be trivial to generate a simple discriminatory test. That is a single marker may be expected to be present at a high frequency in one breed and very low in a second breed, but the difference may not be absolute, and a third breed might have an intermediate frequency. This can be overcome by combining a number of such markers, however, this will require extensive screening of breeds to obtain the best markers. Microsatellite markers are also termed anonymous markers as they do not usually influence the phenotype of the animal. Traits such as coat colour have been used as trademarks by animal breeders for many years. Therefore markers for such traits are ideal for generating simple authenticity tests as described here.

The relationship of the test to coat colour is useful in that it is a tangible concept easily accepted by non specialists. The red colour of the Duroc breed is identified by a single base pair change at position 844 of the αMSHR gene. Animals containing 50% Duroc should therefore contain one copy of this allele and one copy of a second allele. Animals which do not contain one copy of this allele will not meet the "required specification".

Similarly, Wild Boar meat, or meat from crosses involving Wild boar, is marketed in many countries and there is a need for genetic markers which can be used to test that such meat is really from Wild Boar or crosses and not simply from domestic pig breeds. Our invention now provides a simple DNA test which distinguish the Wild Boar from major domestic pig breeds. Such a test will also be of interest for those who breed Wild Boars for meat or game production to ensure the purity of their stock

Thus, in a final aspect the present invention provides a method for authenticating a pig meat product which comprises a method as described herein.

The invention will now be described with reference to the following examples which should not be construed as in any way limiting the invention.

The examples refer to the figures in which:

Figure 1: shows partial nucleotide sequence (a) and the derived amino acid sequence (b) of the porcine αMSHR gene as determined from a number of pig breeds. Position numbers are in accordance with the bovine BDF3 sequence (Vanetti *et al, FEBS Lett.*, **348**:268-272 (1995));

Figure 2: shows agarose gel electrophoresis of DNA fragments obtained by digestion of DNA fragments amplified from the porcine αMSHR gene with *BstUI* or *HhaI*. Lanes labelled M contain DNA markers of 50, 150, 300,500, 750, 1000bp. The other samples were derived from;

1.Pietrain
2.Pietrain
3.Large White
4.Large White
5.Large White
6.Duroc
7.Duroc
8.Hampshire
9.Meishan
10.Berkshire
11.Berkshire ; and

Figure 3: shows agarose gel electrophoresis of DNA fragments obtained by digestion of DNA fragments amplified from the porcine αMSH-R gene in pork chops with *BstUI* (lanes labelled B) or *HhaI* (lanes labelled H). Lanes labelled M contain DNA markers of 50, 150, 300,500, 750, 1000bp. The other samples were derived from:

1.Retailer 1. Muscle
2.Retailer 1. Fat
3.Retailer 1. Rind
4.Retailer 2. Fat
5.Retailer 2. Muscle

Example 1

The DNA sequence of the porcine αMSHR gene was determined

through the DNA sequencing of a combination of PCR products and cloned portions of porcine DNA.

Preparation of template DNA for PCR

DNA can be prepared from any source of tissue containing cell nuclei, for example white blood cells, hair follicles, ear notches and muscle. The procedure here relates to blood cell preparations; other tissues can be processed similarly by directly suspending material in K buffer and then proceeding from the same stage of the blood procedure. The method outlined here produces a cell lysate containing crude DNA which is suitable for PCR amplification. However, any method for preparing purified, or crude, DNA should be equally effective.

Blood was collected in 50mM EDTA pH 8.0 to prevent coagulation. 50µl of blood was dispensed into a small microcentrifuge tube (0.5ml Eppendorf or equivalent). 450µl of TE buffer was added to lyse the red blood cells (haem groups inhibit PCR) and the mix vortexed for 2 seconds. The intact white and residual red blood cells were then centrifuged for 12 seconds at 13,000 g in a microcentrifuge. The supernatant was removed by gentle aspiration using a low pressure vacuum pump system. A further 450µl of TE buffer was then added to lyse the remaining red blood cells and the white blood cells collected by centrifugation as before. If any redness remained in the pellet, this process was repeated until the pellet was white. After removal of the last drop of supernatant from the pelleted white blood cells, 100µl of K buffer containing proteinase K was added and the mixture incubated at 55°C for 2 hours. The mixture was then heated to 95-100°C for 8 minutes and the DNA lysates stored at -20°C until needed.

Reagents

T.E. Buffer:	10mM TRIS-HCl pH8.0
	1mM EDTA
K Buffer:	50mM KCl
	10mM TRIS-HCl pH8.3
	2.5mM $MgCl_2$
	0.5% Tween 20

PCR to produce DNA sequencing template

The αMSHR gene was amplified for sequence analysis using three primer pairs.

Primers MSHR Forward Primer 1: (5'-TGT AAA ACG ACG GCC AGT RGT GCC TGG AGG TGT CCA T-3'); and

MSHR Forward Primer 5: (5'-CGC CCA GAT GGC CGC GAT GGA CCG-3')

amplify a 428 bp fragment from the 5' half of the gene.

Primers MSHR Forward Primer 2: (5'-CGG CCA TCT GGG CGG GCA GCG TGC-3');

and MSHR Reverse Primer 2: (5'-GGA AGG CGT AGA TGA GGG GGT CCA-3')

amplify a 405 bp fragment the 3' half of the gene.

As these two fragments are non-overlapping a third primer pair
MSHR Forward Primer 4 (5'-TGC GCT ACC ACA GCA TCG TGA CCC TGC-3'); and

MSHR Reverse Primer 4 (5'-GTA GTA GGC GAT GAA GAG CGT GCT-3')

were used to amplify a 98 bp fragment which spans the 50

bp gap. PCR was carried out on a DNA thermal cycler (Perkin Elmer 9600) in a total volume of 20 µl containing 25 ng genomic DNA, 1.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 200 µM dNTPs, 0.5 U AmpliTaq Gold (Perkin Elmer) and 10 pmol of both forward and reverse primer. To activate AmpliTaq Gold, initial heat denaturation was carried out at 94°C for 10 minutes followed by 32 cycles each consisting of 45 sec at 94°C, 45 sec at 53°C and 45 sec at 72°C. The final extension lasted for 7 min at 72°C. PCR products were cloned into vector pUC18 using the SureClone ligation kit (Pharmacia).

Preparation of plasmid DNA

Plasmid DNA was purified from overnight bacterial culture using the Jetstar plasmid midi kit 50 (Genomed) and the resulting DNA diluted to 150 ng/µl.

Sequencing of plasmid DNA

Cloned plasmid inserts were sequenced using dye primer chemistry. Each cycling reaction was prepared with template and ready reaction mix containing fluorescently labelled M13 forward or reverse primer as described in the ABI Prism protocol P/N 402113 (Perkin Elmer). Cycling and sample pooling was performed using a Catalyst 800 Molecular Biology Workstation (ABI) following the instruments user manual (Document number 903877, Perkin Elmer). The resulting extension products were purified, loaded and analysed using the 377 ABI Prism DNA sequencer as described by the instrument protocol (Perkin Elmer protocol P/N 402078).

Dye Terminator Sequencing of PCR products

Dye terminator DNA sequencing requires purification of PCR product free from excess dNTPs and residual primers. This was achieved by passage of the template DNA through

QiaQuick spin columns (Qiagen) before the purified DNA was diluted to 15 ng/µl. Dye terminator cycle sequencing was performed using AmpliTaq DNA polymerase FS in accordance with the ABI Prism protocol P/N 402078 (Perkin Elmer). Cycle sequencing reactions were performed in a total reaction volume of 10 µl. This comprised 1.6 pmole of either the forward or reverse primer used to amplify the target fragment from genomic DNA, 20 ng of purified template DNA and terminator ready reaction mix (Perkin Elmer) which contains each of four dye terminators, dNTPs, Tris-HCl (pH 9.0), $MgCl_2$, thermal stable pyrophosphate and AmpliTaq DNA polymerase FS. Cycle sequencing was performed with a GeneAmp 9600 machine (Perkin Elmer) over 25 cycles, each consisting of 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C. Extension products were purified for gel separation using ethanol precipitation, loaded and run on a 377 ABI Prism DNA sequencer as described by the instrument protocol (Perkin Elmer protocol P/N 402078).

Results

The partial coding region DNA sequence of the porcine αMSHR gene sequence from a number of pig breeds is given in figure 1a. The derived amino acid sequence is shown in figure 1b.

Example 2

DNA preparation for PCR

As in example 1.

PCR

Reactions were set up in a 20μl reaction volume in thin walled 0.25ml tubes (Perkin Elmer) with the following components:

20μl reaction volume:
2μl template DNA
1.5 mM $MgCl_2$
200μM each dNTP,
3pM each of forward and reverse primers
0.5 U AmpliTaq Gold (Perkin Elmer)

MSHR Forward primer 3 sequence: 5′ GCA CAT CGC CCG GCT CCA CAA GAC 3′

MSHR Reverse primer 3 sequence: 5′ GGG GCA GAG GAC GAC GAG GGA GAG 3′

The reaction tubes were placed on a Perkin Elmer 9600 thermal cycler preheated to 94°C and PCR carried out according to the regime below: -

Initial denaturation step of 94°C for 10 min.

33 cycles: 94°C - 45 secs
53°C - 45 secs
72°C - 45 secs

The last cycle is followed by a final elongation of 72°C for 7 min. Samples are stored at 0°C until required.

Restriction Enzyme Digestion and Electrophoresis

The PCR amplification product is 148bp in length. To test for polymorphism in the amplified products the reaction is split into two aliquots of 10μl each of which is digested with *Hha*I (GIBCO-BRL) or *Bst*UI (New England Biolabs). The reactions are set up and incubated as

below:

BstUI digest	**HhaI digest**
10μl amplified DNA	10μl amplified DNA
2.5u *Bst*UI	2.5u *Hha* I
60°C 60 minutes	0.5ml 10x React 2 buffer (GIBCO-BRL)
	37°C 60 minutes

Following digestion, 2μl of loading dye is added to each reaction (100mM Tris pH8.0, 100mM Boric Acid, 1mM EDTA, 50% (v/v) glycerol, 0.02% w/v Orange G) and the mixes loaded on a 4% agarose gel (3% NuSieve/1% Seakem, FMC Bioproducts) in 0.5x TBE (44.5mM Tris pH8.0, 44.5 mM boric acid and 0.5mM EDTA) and electrophoresed for 1 hour at 150v.

Products are visualised by ethidium bromide staining.

Results

*Bst*UI and *Hha*I digestion each result in bands of 61 and 87bp. The relationship of digestion to the possible allele is as shown in table 1

Table 1. Relationship of restriction digest profiles to individual alleles at the *E* locus

Allele	Digestion with *Bst*UI	Digestion with *Hha*I
$E^{-}/E^{-}/E^{-}$	Yes	Yes
E^{m}	No	Yes
e	No	No

If the uncut alleles are designated as allele 1 and the alleles digesting with each enzyme as allele 2 the

various genotypes will be as shown below:

Table 2. Actual *E* genotypes and associated scores

Genotype	*Bst*UI	*Hha*I
E^m/E^m	1/1	2/2
E^m/E^p or E^m/E^+	1/2	2/2
E^m/e	1/1	1/2
E^p/E^p or E^p/E^+ or E^+/E^+	2/2	2/2
E^p/e or E^+/e	1/2	1/2
e/e	1/1	1/1

Note: The results for animals carrying the allele E^h will be the same as those carrying E^p.

Samples were prepared from a number of pigs and tested according to the above protocol. The results are shown in table 3 and figure 2 illustrates the patterns seen upon electrophoresis.

Table 3. *E* genotypes determined for a range of breeds using the *Bst*UI/*Hha*I digestion system

Breed	No Tested	Genotype (see note 1)	αMSHR type *Bst*UI	αMSHR type *Hha*I
Hampshire	9	E^p/E^p	2/2	2/2
Large White	4	E^p/E^p	2/2	2/2
Landrace	1	E^p/E^p	2/2	2/2
Pietrain	3	E^p/E^p	2/2	2/2
Berkshire	2	E^p/E^p	2/2	2/2
Bazna	4	E^p/E^m	1/2	2/2
		E^m/E^m	1/1	2/2
Duroc	4	e/e	1/1	1/1
Meishan	3	E^m/E^m	1/1	2/2

Note 1. Where the genotype E° is listed this cannot be distinguished from E^{+} or E^{n}.

As can be seen from the results above the genotypes determined fit with those expected from the sequencing data given in figure 1a for Hampshire, Large White, Meishan and Duroc. The additional breeds typed here show the genotypes expected from their phenotype and descriptions in published literature (Ollivier and Sellier, *Ann. Génét. Sél. anim.*, **14**: 481-544, (1982)). The Pietrain is a white breed with black patches of varying extent and has long been considered to be E° (in agreement with the result here). The Berkshire, originally a spotted breed, is now a mainly black animal with white 'socks' again generally considered to be E° as was found here. The Landrace is a white animal due to it carrying the dominant white allele at the *I* locus, however its genotype at the *E* locus has been shown to be E° from classical breeding studies. Once again this is in agreement with the results obtained here. The Bazna is a Romanian breed having black base colour with a white belt. It was developed from the Berkshire and Mangalitza, a Hungarian breed with a number of colour variations including black (Porter, *Pigs, a handbook to breeds of the world*, publ: Helm Information, ISBN 1-873403-17-8 (1993)). The ancestory of the Bazna being based upon a black breed potentially carrying a similar allele to the Meishan, E^{n}, and the Berkshire carrying E°, is in agreement with the alleles found to be present in the breed in this work.

Example 3

DNA preparation

DNA was prepared from different parts of pork chops from two separate retailers. The DNA was prepared from skin (1 retailer only), fat and muscle using the Promega

Wizard Genomic DNA preparation kit according to the manufacturers instructions. Approximately 4mm^3 of each tissue was cut into small fragments for the extraction.

PCR and Restriction Digest Analysis

This was carried out exactly as in example 2

Results

The results are shown in figure 3. It can be seen that DNA extracted from a range of tissue types can be utilised for this DNA based test with results being obtained here for muscle, fat and skin. The genotype of the pig with regard to the αMSHR gene can then be determined. In this case the material from both retailers was derived from an animal of test type *Bst*UI 1/2 and *Hha*I 1/2 using the nomenclature as in example 2. This translates into genotype E^D/e or E^r/e. Based on our current knowledge of the distribution of the alleles in commercial pig breeds the conclusion can be drawn that both source animals contain genetic material derived from the Duroc.

CLAIMS:

1. A method of determining the coat colour genotype of a pig which comprises:

(i) obtaining a sample of pig nucleic acid; and

(ii) analysing the nucleic acid obtained in (i) to determine which allele or alleles of the αMSHR gene are present.

2. A method as claimed in claim 1 wherein the determination in step (ii) is carried out by determining the nucleotide sequence of the αMSHR gene.

3. A method as claimed in claim 1 or claim 2 wherein the determination in step (ii) involves identifying the presence or absence of at least one missense mutation in the coding region of the αMSHR gene.

4. A method as claimed in claim 1 which also includes the step determining the association between one or more microsatellite marker alleles linked to the αMSHR gene and particular alleles of the αMSHR gene.

5. A method as claimed in claim 4 wherein the determination in step (ii) is based on identification of microsatellite markers present in the nucleic acid sample.

6. A method of determining the coat colour genotype of a pig which comprises:

(i) determining the association between one or more microsatellite marker alleles linked to the αMSHR gene and particular alleles of the

αMSHR gene;

(ii) obtaining a sample of pig nucleic acid; and

(iii) analysing the nucleic acid obtained in (ii) to determine which microsatellite marker allele or alleles are present.

7. A method as claimed any one of claims 1 to 6 which further comprises the step of determining the genotype of at least one additional locus.

8. A method as claimed in claim 7 wherein the at least one additional locus is an additional coat colour locus.

9. A method as claimed in claim 8 wherein the additional coat colour locus is the *KIT* gene locus.

10. A method as claimed in claim 7 wherein the at least one additional locus is a gene for a specific breed characteristic.

11. A method as claimed in claim 7 wherein the at least one additional locus is a breed specific marker.

12. A method as claimed in claim 11 wherein the breed specific marker is a microsatellite marker.

13. A method as claimed in any one of claims 1 to 12 wherein the pig nucleic acid is pig genomic DNA.

14. A method as claimed in any one of claims 1 to 13 wherein the determination in step (ii) or step (iii) is carried out using PCR, using at least one pair of suitable primers.

15. A method as claimed in claim 14 wherein the at least one pair of suitable primers is:

MSHR Forward primer 1 sequence: 5′ CGG CCA TCT GGG CGG GCA GCG TGC- 3′

and

MSHR Reverse primer 1 sequence: 5′ CAG GAA ACA GCT ATG ACC GTC MTG CGG AGC CTC CTG GCT- 3′; or

MSHR Forward primer 2 sequence: 5′ CGG CCA TCT GGG CGG GCA GCG TGC- 3′

and

MSHR Reverse primer 2 sequence: 5′ GGA AGG CGT AGA TGA GGG GGT CCA- 3′

or

MSHR Forward primer 3 sequence: 5′ GCA CAT CGC CCG GCT CCA CAA GAC- 3′

and

MSHR Reverse primer 3 sequence: 5′ GGG GCA GAG GAC GAC GAG GGA GAG- 3′.

16. A method as claimed in any one of claims 1 to 15 wherein the determination carried out in step (ii) or step (iii) comprises a Restriction fragment Length Polymorphism (RFLP) analysis.

17. A method as claimed in claim 16 wherein the determination involves identification of a polymorphism at nucleotide position 400, 422, 480, 487, 608, 844, 846 or in the region 482-487 or 841-849 of the coding sequence of the αMSHR gene.

18. A method of determining the coat colour genotype of a pig which comprises:

(i) obtaining a sample of pig nucleic acid;

(ii) carrying out one or more PCR amplification cycles of the nucleic acid obtained in step (i) using at least one pair of suitable primers; and

(iii) carrying out RFLP analysis on the amplified nucleic acid obtained in step (ii) to determine the αMSHR genotype of the pig.

19. A method as claimed in claim 18 wherein the at least one pair of suitable primers is:

MSHR Forward primer 1 sequence: 5′ CGG CCA TCT GGG CGG GCA GCG TGC- **3′**

and

MSHR Reverse primer 1 sequence: 5′ CAG GAA ACA GCT ATG ACC GTC MTG CGG AGC CTC CTG GCT- **3′**; or

MSHR Forward primer 2 sequence: 5′ CGG CCA TCT GGG CGG GCA GCG TGC- **3′**

and

MSHR Reverse primer 2 sequence: 5′ **GGA AGG CGT AGA TGA GGG GGT CCA-** 3′

or

MSHR Forward primer 3 sequence: 5′ GCA CAT CGC CCG GCT CCA CAA GAC- **3′**

and

MSHR Reverse primer 3 sequence: 5′ GGG GCA GAG GAC GAC GAG GGA GAG- 3′.

20. A method as claimed in claim 18 or claim 19 wherein the RFLP analysis involves identification of a polymorphism at nucleotide position 400, 422, 480, 487, 608, 844, 846 or in the region 482-487 or 841-849 of the coding sequence of the αMSHR gene.

21. A method as claimed in any one of claims 16 to 20 wherein the RFLP analysis involves digesting the pig nucleic acid with one or more of the restriction enzymes *Bst*UI, *Hha*I or *Bsp*HI.

22. A method as claimed in any one of claims 18 to 21 wherein the nucleic acid obtained in step (i) is pig genomic DNA.

23. A method as claimed in any one of claims 18 to 22 modified by any one or more of the features of claims 7 to 12.

24. A method of determining the coat colour genotype of a pig which comprises:

(i) obtaining a sample of pig αMSHR protein; and

(ii) analysing the protein obtained in step (i) to determine the amino acid sequence at the variant positions associated with coat colour genotype.

25. A kit for determining the coat colour genotype of a pig, comprising one or more reagents for analysing the αMSHR genotype of the pig.

26. A kit as claimed in claim 25 wherein the kit is adapted to be used with a sample of pig genomic DNA.

27. A kit as claimed in claim 25 or claim 26 comprising one or more reagents for carrying out at least one cycle of PCR together with at least one pair of suitable primers.

28. A kit as claimed in claim 27 wherein the at least one pair of suitable primers is:

MSHR Forward primer 1 sequence: 5′ CGG CCA TCT GGG CGG GCA GCG TGC- 3′

and

MSHR Reverse primer 1 sequence: 5′ CAG GAA ACA GCT ATG ACC GTC MTG CGG AGC CTC CTG GCT- 3′; or

MSHR Forward primer 2 sequence: 5′ CGG CCA TCT GGG CGG GCA GCG TGC- 3′

and

MSHR Reverse primer 2 sequence: 5′ **GGA AGG CGT AGA TGA GGG GGT CCA-** 3′

or

MSHR Forward primer 3 sequence: 5′ GCA CAT CGC CCG GCT CCA CAA GAC- 3′

and

MSHR Reverse primer 3 sequence: 5′ GGG GCA GAG GAC GAC GAG GGA GAG- 3′.

29. A kit as claimed in any one of claims 25 to 28 which comprises one or more reagents for RFLP analysis of pig nucleic acid.

30. A method for the authentication of a pig meat product which comprises a method as defined in any one of claims 1 to 24.

Figure 1

a: Nucleotide sequence

```
                                                                                                                294
Wildboar    ??C CCG AAC GGG CTC TTC CTC AGC CTG GGG CTG GTG AGC CTC GTG GAG AAC
Meishan     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Duroc       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                                                                345
Wildboar    GTG CTG GTG GTG GCC GCC ATC GCC AAG AAC CGC AAC CTG CAC TCG CCC ATG
Meishan     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Duroc       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                                                                396
Wildboar    TAC TAC TTC GTC TGC TGC CTG GCC GTG TCG GAC CTG CTG GTG AGC GTG AGC
Meishan     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Duroc       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                                                                447
Wildboar    AAC GTG CTG GAG ACG GCC GTG CTG CTG CTG CTG GAG GCG GGC GCC CTG GCC
Meishan     ... A.. ... ... ... ... ... ... .C. ... ... ... ... ... ... ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Duroc       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                                                                498
Wildboar    GCC CAG GCC GCC GTG GTG CAG CAG CTG GAC AAT GTC ATG GAC GTG CTC ATC
Meishan     ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ...
Duroc       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                                                                549
Wildboar    TGC GGC TCC ATG GTG TCC AGC CTC TGC TTC CTG GGC GCC ATC GCC GTG GAC
Meishan     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Duroc       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                                                                600
Wildboar    CGC TAC GTG TCC ATC TTC TAC GCG CTG CGC TAC CAC AGC ATC GTG ACG CTG
Meishan     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Duroc       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
```

Figure 1 continued

```
                                                                                                                651
Wildboar    CCC CGC GCG GGG CGG GCT ATC GCG GCG ATC TGG GCG GGC AGC GTG CTC TCC
Meishan     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Duroc       ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                                                                702
Wildboar    AGC ACC CTC TTC ATC GCC TAC TAC CAC CAC ACG GCC GTC CTG CTG GGC CTC
Meishan     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Duroc       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                                                                753
Wildboar    GTC AGC TTC TTC GTG GCC ATG CTG GCG CTC ATG GCG GTA CTG TAC GTC CAC
Meishan     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Duroc       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                                                                804
Wildboar    ATG CTG GCC CGG GCC TGC CAG CAC GGC CGG CAC ATC GCC CGG CTC CAC AAG
Meishan     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Duroc       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                                                                855
Wildboar    ACG CAG CAC CCC ACC CGC CAG GGC TGC GGC CTC AAG GGC GCG GCC ACC CTC
Meishan     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Duroc       ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ...

                                                                                                                906
Wildboar    ACC ATC CTG CTG GGC GTC TTC CTC CTC TGC TGG GCA CCC TTC TTC CTG CAC
Meishan     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Duroc       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                                                                957
Wildboar    CTC TCC CTC GTC GTC CTC TGC CCC CAG CAC CCC ACC TGC GGC TGC GTC TTC
Meishan     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Largewhite  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Hampshire   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Duroc       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
```

Figure 1 continued

Wildboar	AAG	AAC	GTC	AAC	CTC	TTT	CTG	GCC	CTC	GTC	ATC	TGC	AAC	TCC	ATC
Meishan	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Largewhite	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Hampshire	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Duroc	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

Figure 1 continued

b. Amino acid sequence

```
                                                                                                    99
Wildboar    ?PNGLFLSLG LVSLVENVLV VAAIAKNRNL HSPMYYFVCC LAVSDLLVSV SNVLETAVLL
Meishan     .......... .......... .......... .......... .......... ..M......P
Largewhite  .......... .......... .......... .......... .......... ..........
Hampshire   .......... .......... .......... .......... .......... ..........
Duroc       .......... .......... .......... .......... .......... ..........

                                                                                                   159
Wildboar    LLEAGALAAQ AAVVQQLDNV MDVLICGSMV SSLCFLGAIA VDRYVSIFYA LRYHSIVTLP
Meishan     .......... .......... .......... .......... .......... ..........
Largewhite  .......... .......... .N........ .......... .......... ..........
Hampshire   .......... .......... .N........ .......... .......... ..........
Duroc       .......... .......... .......... .......... .......... ..........

                                                                                                   219
Wildboar    RAGRAIAAIW AGSVLSSTLF IAYYHHTAVL LGLVSFFVAM LALMAVLYVH MLARACQHGR
Meishan     .......... .......... .......... .......... .......... ..........
Largewhite  .......... .......... .......... .......... .......... ..........
Hampshire   .......... .......... .......... .......... .......... ..........
Duroc       .V........ .......... .......... .......... .......... ..........

                                                                                                   279
Wildboar    HIARLHKTQH PTRQGCGLKG AATLTILLGV FLLCWAPFFL HLSLVVLCPQ HPTCGCVFKN
Meishan     .......... .......... .......... .......... .......... ..........
Largewhite  .......... .......... .......... .......... .......... ..........
Hampshire   .......... .......... .......... .......... .......... ..........
Duroc       .......... .......... T......... .......... .......... ..........

Wildboar    VNLFLALVIC NSI
Meishan     .......... ...
Largewhite  .......... ...
Hampshire   .......... ...
Duroc       .......... ...
```

Figure 2

*Bst*UI

*Hha*I

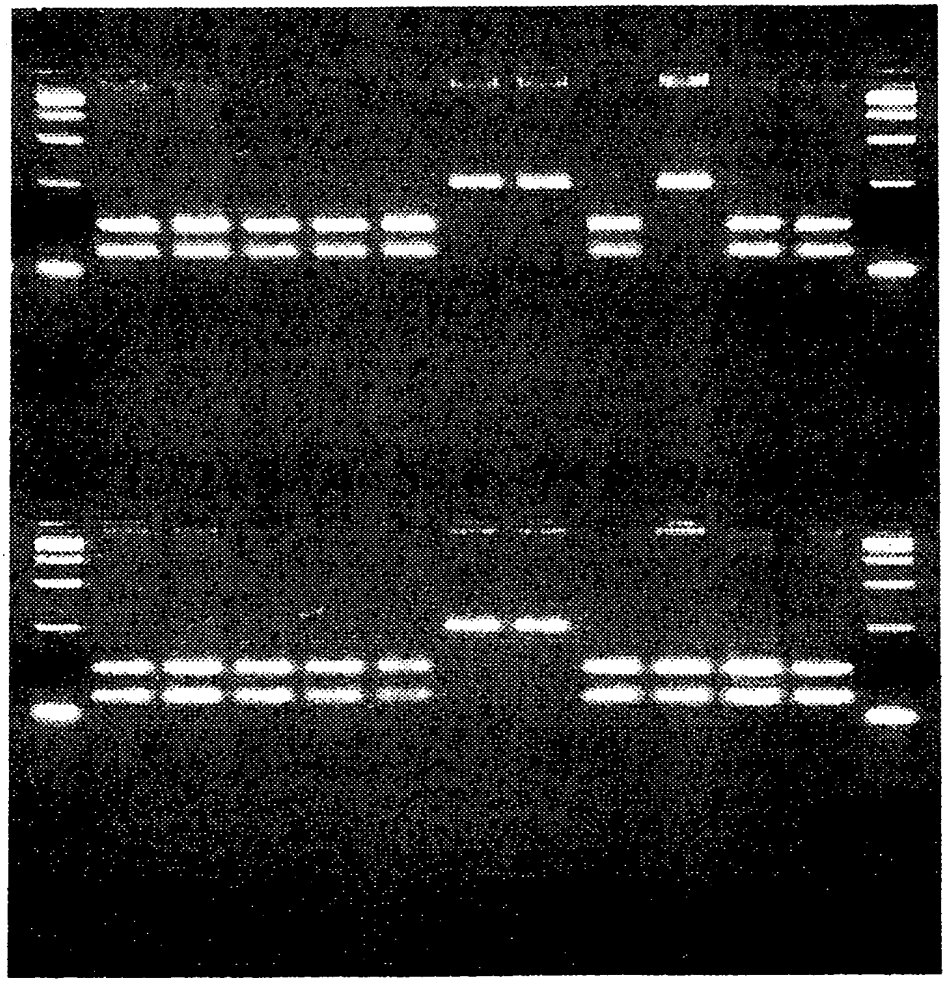

Figure 3